Omega-3 PUFA of Marine Origin Limit Diet-Induced Obesity in Mice by Reducing Cellularity of Adipose Tissue

Jana Ruzickova[a], Martin Rossmeisl[a], Tomas Prazak[a], Pavel Flachs[a], Jana Sponarova[a], Marek Vecka[b], Eva Tvrzicka[b], Morten Bryhn[c], and Jan Kopecky[a,*]

[a]Department of Adipose Tissue Biology and Centre for Integrated Genomics, Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic, [b]4th Department of Medicine,1st Medical Faculty, Charles University, 128 08 Prague, Czech Republic, and [c]Pronova Biocare a.s., N-1327 Lysaker, Norway

ABSTRACT: Omega-3 PUFA of marine origin reduce adiposity in animals fed a high-fat diet. Our aim was to learn whether EPA and DHA could limit development of obesity and reduce cellularity of adipose tissue and whether other dietary FA could influence the effect of EPA/DHA. Weight gain induced by composite high-fat diet in C57BL/6J mice was limited when the content of EPA/DHA was increased from 1 to 12% (wt/wt) of dietary lipids. Accumulation of adipose tissue was reduced, especially of the epididymal fat. Low ratio of EPA to DHA promoted the effect. A higher dose of EPA/DHA was required to reduce adiposity when admixed to diets that did not promote obesity, the semisynthetic high-fat diets rich in EFA, either α-linolenic acid (ALA, 18:3 n-3, the precursor of EPA and DHA) or linoleic (18:2 n-6) acid. Quantification of adipose tissue DNA revealed that except for the diet rich in ALA the reduction of epididymal fat was associated with 34–50% depression of tissue cellularity, similar to the 30% caloric restriction in the case of the high-fat composite diet. Changes in plasma markers and adipose gene expression indicated improvement of lipid and glucose metabolism due to EPA/DHA even in the context of the diet rich in ALA. Our results document augmentation of the antiadipogenic effect of EPA/DHA during development of obesity and suggest that EPA/DHA could reduce accumulation of body fat by limiting both hypertrophy and hyperplasia of fat cells. Increased dietary intake of EPA/DHA may be beneficial regardless of the ALA intake.

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Many studies in rodents have demonstrated that PUFA, especially n-3 PUFA EPA (20:5n-3) and DHA, 22:6n-3, which are abundant in marine fish oils, are less effective in promoting accumulation of adipose tissue than saturated fats (1–5). Dietary n-3 PUFA admixed to high-fat (HF) diets do not affect food consumption (2,6–8), but they do modulate fuel partitioning by downregulating lipogenesis and stimulating lipid oxidation. Such modulation of metabolism is associated with changes of gene expression in many tissues including liver, muscle, and adipose tissue (4,9,10). A preferential reduction of epididymal compared with subcutaneous white adipose tissue due to enrichment of diet with fish oil or EPA/DHA concentrates was observed (2,7,10,11). However, the mechanism for the reduction of body fat stores is still unclear [see the following discussion and Azain (4), Lapillonne *et al.* (9), and Raclot and Oudart (10)]. Both in animals (6,10) and humans (12,13), EPA/DHA lower blood TG and may improve insulin sensitivity. They also exert prophylactic effects on cardiovascular disease (12,14). Data on the effects of n-3 PUFA on adiposity in humans are scarce (13,15).

The effect of EPA/DHA on adiposity was mostly studied in rats using semisynthetic HF (sHF) diets and compared with saturated FA or n-6 PUFA (1,2,5,10,11,16,17). Especially DHA-rich diets were potent in reducing body fat storage, and the reduction was explained by limited accumulation of lipids in adipocytes rather than by a decreased number of fat cells (2,11). A drawback of the above studies was that they were not conducted under conditions promoting obesity, since the body weight gain of control animals was very small. Also studies in mice fed sHF diets have documented the reduction of adiposity by n-3 PUFA (6,7,18,19) and indicated that EPA and DHA could be more effective than n-3 PUFA of plant origin, that is, α-linolenic acid [ALA, 18:3 n-3; Ikemoto *et al.* (6)]. This compound is a precursor of EPA and DHA in mammals, but it is oxidized very rapidly in the organism and its conversion to EPA and DHA is quite inefficient (4,12,14).

Inbred and genetically modified strains of mice are being increasingly used to study the pathophysiology of obesity and its related disorders. For instance, the C57BL/6J inbred strain of mice represents a common model of obesity and diabetes induced by composite HF diets [cHF diets (20–23)]. The previous mouse strain has been already used to study the effects of different dietary oils admixed to sHF diets on body fat accumulation and glucose metabolism (6). The goal of this study was to learn whether EPA and DHA could limit the obesity and proliferation of adipose tissue cells induced in the C57BL/6J mice by the cHF diet. To understand the influence of other dietary lipids, namely EFA of plant origin (i.e., 18:3n-3 and 18:2n-6), on the potency of EPA and DHA to reduce the mass and cellularity of adipose tissue, experiments were also

*To whom correspondence should be addressed at Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, 142 20 Prague, Czech Republic. E-mail: kopecky@biomed.cas.cz

Abbreviations: ALA, α-linolenic acid; CR, caloric restriction by 30% compared with *ad libitum* fed mice; HF, high-fat; cHF diet, composite high-fat diet; cHF-F1 and cHF-F2, composite high-fat diets enriched with fish oil concentrate; sHFc, semisynthetic high-fat diet based on corn oil; HFc-F1, sHFc-F2, semisynthetic high-fat diets based on corn oil enriched with fish oil concentrate; sHFf, semisynthetic high-fat diet based on flaxseed oil; sHFf-F1, sHFf-F2, semisynthetic high-fat diets based on flaxseed oil enriched with fish oil concentrate; NEFA, nonesterified FA; and sHF diet, semisynthetic high-fat diet.

performed using sHF diets based on flaxseed or corn oils. The effects on gene expression in adipose tissue, as well as the plasma levels of lipids, leptin, and insulin in the mice fed sHF diets were also analyzed.

EXPERIMENTAL PROCEDURES

Animals and diets. Male mice were housed in a controlled environment (20°C; 12-h light/dark cycle; light from 6:00 a.m.) with free access to water and a standard chow diet [Velaz, Prague, Czech Republic; Kopecky *et al.* (22)]. At the age of 2.5 mon mice were single-caged. At 3 to 4 mon of age, the animals were randomly assigned to experimental HF diets: (i) obesity-promoting cHF diet derived from the standard chow (20,22); or (ii) sHF diet of a similar composition like the one previously used for rats (2,11), containing (in g/kg) 479 sucrose, 266 casein, 200 plant oil [either flaxseed oil rich in ALA, 18:3 n-3; or corn oil rich in linoleic acid, 18:2 n-6; Table 2], 45 agar-agar, 10 vitamin and mineral mix (Biofaktory, Prague, Czech Republic), and α-tocopherol (300 mg/kg). Macronutrient composition and energy density of the chow, cHF, and sHF diets are described in Table 1. When indicated, 15 or 44% (wt/wt) of a fat component in the HF diets was replaced by the concentrate of n-3 PUFA of marine origin rich in DHA (EPAX 1050 TG or EPAX 2050 TG) or EPA (EPAX 4510 TG; Pronova Biocare a.s., Lysaker, Norway). EPA and DHA collectively form 45–64% (wt/wt) of the concentrates and they are present as TG. The lipid composition and abbreviations of diets are given in Table 2. FA composition of all diets used in this study was determined by GC (Table 3). Mice were weighed weekly, while individual food intake was determined daily, when a new ration was given. In some experiments, the ration was reduced by 30% compared with *ad libitum* fed mice on the same type of diet (caloric restriction; CR). At the end of the feeding study, at 5 to 6.5 mon of age, mice were killed by cervical dislocation between 9:00 and 10:00 a.m., subcutaneous (dorsolumbar) and epididymal white fat [for the anatomical description of fat depots, see Cinti (24)], as well as the liver, were dissected. Plasma was obtained from truncal blood using EDTA and stored at –70·C for the analysis of metabolites and hormones (see the following discussion).

TABLE 1
Macronutrient Composition and Energy Density of Experimental Diets

	Diet		
	Chow	cHF	sHF
Lipids (% diet, wt/wt)	3.4	35.2	20.0
Carbohydrates (% diet, wt/wt)	56.2	35.4	47.9
Proteins (% diet, wt/wt)	20.9	20.5	26.6
Energy density (kJ/g)	14.2	22.8	10.1

Tissue content of TG and DNA and TG in feces. Content of TG in liver and feces was estimated in alcoholic KOH solubilizates prepared according to Salmon and Flatt (25), using a kit (Catalog No. 320-A) from Sigma Diagnostic (Procedure No.320-UV, Sigma, St. Louis, MO), as described previously (22). DNA was estimated fluorometrically in tissue samples digested with proteinase K as before (26).

Plasma concentrations of metabolites and hormones. Insulin, leptin, and TG were estimated in plasma of fed animals (22). Concentrations of nonesterified FA (NEFA) in plasma were evaluated enzymatically using a NEFA C kit (Wako Chemicals, Richmond, CA). Plasma leptin and insulin were assessed by using a Mouse Leptin RIA Kit and Sensitive Rat Insulin RIA Kit, respectively, from LINCO Research (St. Charles, MO). Blood glucose was measured by the use of a SmartScan glucometer (Life Scan, Milpitas, CA).

Gene expression. Total RNA was isolated by using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was treated with RNase-free DNase. Gene expression was analyzed by reverse transcription followed by the real-time quantitative PCR (LightCycler Instrument, F. Hoffman-La Roche Ltd., Basel,

TABLE 2
Lipid Composition and Abbreviations of Experimental Diets[a]

	Oil (% lipid, wt/wt)						
Diet	Rapeseed	Sunflower	Flaxseed	Corn	EPAX 1050TG	EPAX 2050TG	EPAX 4510TG
Control diets							
cHF[b]	95	5	—	—	—	—	—
sHFf	—	—	100	—	—	—	—
sHFc	—	—	—	100	—	—	—
EPA/DHA-enriched diets							
EPA < DHA[c]							
cHF-F1[b]	81	4	—	—	15	—	—
cHF-F2[b]	53	3	—	—	44	—	—
sHFf-F1	—	—	85	—	—	15	—
sHFf-F2	—	—	56	—	—	44	—
sHFc-F1	—	—	—	56	44	—	—
EPA > DHA[c]							
sHFc-F2	—	—	—	56	—	—	44

[a]Lipid composition of all experimental diets prepared at the laboratory.
[b]The composition of plant oils used to prepare the cHF diets [see Kopecky *et al.* (22) for further details].
[c]EPA-to-DHA ratio in diet.

TABLE 3
FA Composition of Dietary Lipids[a]

	Control diets			EPA/DHA-enriched diets						
FA (g/100 g)	Chow	cHF	sHFf	sHFc	cHF-F1	cHF-F2	sHFf-F1	sHFf-F2	sHFc-F1	sHFc-F2
12:0	0.1	1.5	—	—	1.5	1.5	NE	NE	—	NE
14:0	1.3	2.4	—	—	2.4	2.4	—	0.1	—	0.2
14:1n-5	—	0.2	—	—	0.2	0.2	NE	NE	—	NE
16:0	20.9	11.3	4.2	10.3	10.8	9.8	3.7	2.6	6.3	6.4
16:1n-9	0.2	0.1	—	—	0.1	0.1	0.1	0.2	—	0.3
16:1n-7	1.9	0.5	—	0.1	0.6	0.6	NE	NE	0.2	NE
18:0	6.7	3.7	3.1	1.8	3.9	4.2	3.0	2.7	2.3	3.8
18:1n-9	23.3	50.8	18.1	28.5	42.3	25.9	15.9	11.5	18.9	21.3
18:1n-7	1.8	2.5	0.6	0.5	2.2	1.7	0.6	0.7	0.7	2.3
18:2n-6	32.0	19.4	14.9	52.7	16.6	11.1	12.7	8.6	31.5	30.1
18:3n-6	0.1	—	—	0.1	—	0.1	NE	NE	0.1	0.4
18:3n-3	3.3	4.1	57.9	1.6	3.3	1.9	49.3	32.7	1.1	2.6
18:4n-3	0.4	0.1	—	—	0.2	0.3	0.2	0.6	0.1	NE
20:0	0.2	0.4	0.1	0.4	0.5	0.5	NE	NE	0.5	NE
20:1n-9	0.7	0.9	0.1	3.6	0.9	0.9	0.4	1.0	2.5	4.1
20:2n-6	0.1	0.2	—	0.1	0.2	0.3	0.1	0.2	0.2	NE
20:3n-6	—	—	—	0.1	—	0.1	NE	NE	0.1	NE
20:4n-6	0.2	0.1	—	—	0.4	1.0	0.2	0.5	0.9	1.4
20:3n-3	0.1	—	—	—	—	0.1	NE	NE	0.1	NE
20:4n-3	0.1	—	—	—	0.1	0.2	0.3	0.8	0.2	1.1
20:5n-3	2.4	0.2	—	—	1.3	3.3	3.6	10.6	3.0	21.3
22:0	0.2	0.3	0.1	0.1	0.3	0.3	NE	NE	0.2	NE
22:1n-9	0.1	0.2	—	—	0.2	0.2	0.1	0.2	0.1	0.4
22:4n-6	—	—	—	—	0.2	0.4	NE	NE	0.3	NE
22:5n-6	0.1	0.1	—	—	0.8	2.2	0.2	0.5	2.0	NE
22:5n-3	0.4	0.1	0.2	—	0.4	1.1	0.9	2.4	1.0	0.7
22:6n-3	2.8	0.9	—	—	10.5	29.2	8.0	23.6	27.0	3.7
24:0	0.2	—	0.1	—	0.1	0.1	NE	NE	—	NE
24:1n-9	0.2	0.2	0.3	0.1	0.3	0.6	NE	NE	0.6	NE
Sum SFA	29.7	19.6	7.7	12.6	19.3	18.7	7.0	5.7	9.3	10.6
Sum MUFA	28.3	55.2	19.2	32.8	46.7	30.1	17.3	13.8	23.0	28.4
Sum n-6 PUFA	32.5	19.8	14.9	52.9	18.2	15.2	13.2	9.8	35.2	31.0
Sum n-3 PUFA	9.6	5.4	58.1	1.7	15.8	36.1	62.4	70.7	32.5	29.7

[a]SFA, saturated FA; MUFA, monounsaturated FA; —, <0.1%; NE, not estimated.

Switzerland) with primers specific for mouse leptin (F: CCGCCAAGCAGAGGGTCAC; R: GCATTCAGGGCTAACATCCAACT) and GLUT4 (F: ACCGGCTGGGCTGATGTGTCT; R: GCCGACTCGAAGATGCTGGTTGAATAG). Expression levels of eukaryotic translational elongation factor-1α (F: TGACAGCAAAAACGACCCACCAAT; R: GGGCCATCTTCCAGCTTCTTACCA) and cyclophilin-β (F: ACTACGGGCCTGGCTGGGTGAG; R: TGCCGGAGTCGACAATGATGA) mRNA were used to correct for inter-sample variations with similar results. Expression of these two genes was not affected by the dietary treatments (not shown). The detailed protocol has been described before (27).

Statistics. The data were analyzed by a one-way (diet) or a two-way (depot × diet) ANOVA, using SigmaStat statistical software. Logarithmic transformation was used to stabilize variance in cells when necessary. The Student–Newman–Keuls, or Dunnett's method, applied to separate variance t-test between pairs of groups, was used for multiple comparisons. All comparisons were judged to be significant at $P = 0.05$.

RESULTS

Reduction of cHF diet-induced obesity by EPA/DHA. Adult mice offered a control cHF diet started to gain body weight at a much higher rate than their littermates maintained on the chow diet. Weight gain was apparent after about 2 wk of habituation on the cHF diet and reached about 6.5 g within the next 5 wk of the experiment (Fig. 1). Body weight gain was about 2.7 g lower in mice fed a cHF-F1 diet enriched with EPA/DHA compared with cHF diet (for diets, see Tables 2 and 3) and even higher enrichment resulted in a net loss of body weight of about 3 g over the 5-wk feeding period (Fig. 1, the cHF-F2 mice). Weight loss of about 6 g was observed in food restricted (CR) animals fed the control cHF diet. However, a combination of CR and the cHF-F1 diet had no further effect on body weight compared with CR alone (Fig. 1). The intake of EPA/DHA did not affect food consumption of the animals (Table 4, cHF *vs.* cHF-F2).

A dose-dependent effect of dietary EPA/DHA on body weight could be attributed to a reduced adiposity of both epi-

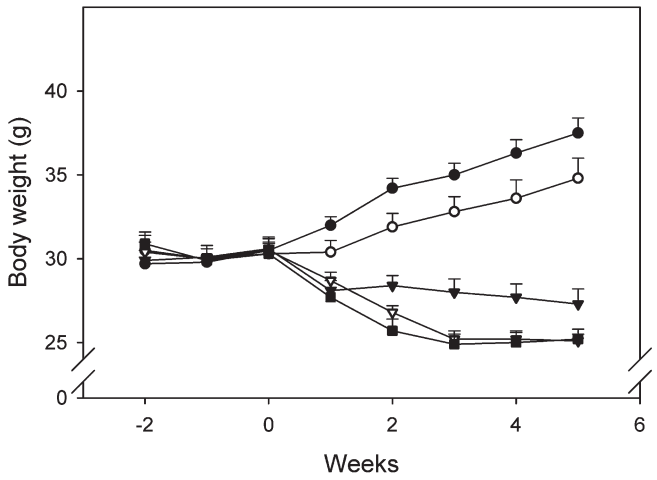

FIG 1. Effects of EPA/DHA and caloric restriction (CR) on body weight and fat accumulation in mice fed a composite high-fat (cHF) diet. At 4 mon of age (wk –2), the chow diet was replaced by the control cHF diet. Two weeks later (week 0), animals were divided into 5 subgroups and fed *ad libitum* the control cHF diet (●), cHF-F1 diet (○), or cHF-F2 diet (▼). Mice on the CR regime were fed either the control cHF diet (▽) or the cHF-F1 diet (■). After 5 wk, the animals were sacrificed. Data are means ± SE (n = 10). See also Table 4.

didymal and subcutaneous (dorsolumbar) fat depots. However, the former depot appeared to be more affected, and even the lower dose of EPA/DHA was effective (Table 4, cHF *vs.* cHF-F1). In fact, both higher dose of EPA/DHA (cHF-F2 diet) and CR reduced adiposity of the depots to a similar extent. No further decrease of fat content was observed when cHF-F1 diet was combined with CR (Table 4). Only CR but not EPA/DHA decreased liver mass (Table 4).

DNA concentration was higher in the dorsolumbar than epididymal fat and increased about 2-fold in both fat depots due to the higher dose of EPA/DHA (Table 4, cHF *vs.* cHF-F2) while the lower dose had no effect (Table 4, cHF *vs.* cHF-F1). In epididymal but not dorsolumbar fat the total DNA amount decreased about 2-fold due to both cHF-F1 and cHF-F2 diets. CR, either alone or in combination with the cHF-F1 diet, increased DNA concentration in both fat depots similarly as the cHF-F2 diet (Table 4). CR alone resulted in about 2-fold depression of total DNA in epididymal fat, while it did not affect DNA content of dorsolumbar fat. The combination of CR and cHF-F1 diet tended to decrease total DNA content in both depots, compared with the effect of CR alone (Table 4).

Depression of adiposity by EPA/DHA admixed to sHF diets. To understand whether the potency of n-3 PUFA of marine origin to reduce adiposity depends on the composition of the bulk of dietary lipids, experiments were performed using the sHF diets based on either flaxseed (sHFf diets) or corn oil (sHFc diets; see Tables 2 and 3). Control sHFf diet did not promote obesity during 2 mon of the experiment and the animals gained similar body weight as those maintained on a chow diet (Table 5, sHFf). The moderate enrichment of the diet with EPA/DHA had no significant effect on body weight; however, the weight of both epididymal and dorsolumbar fat depots tended to increase whereas DNA concentration decreased (Table 5, sHFf *vs.* sHFf-F1). The higher enrichment of the diet with EPA/DHA elicited significant reduction in body weight and adipose tissue mass as well as increase of DNA concentration in epididymal but not dorsolumbar adipose tissue (Table 5, sHFf *vs.* sHFf-F2). However, total content of DNA in neither fat depot was changed.

The next experiment was performed using the sHFc diets, that is, the control sHFc diet and the diets enriched with EPA/DHA, containing either more DHA (as in all the experiments described above) or more EPA (Table 6, sHFc-F1 and sHFc-F2, respectively). Compared to chow diet (not shown), 2.5 mon of feeding the sHFc diet did not affect body weight or mass of fat depots. However, treatment by both sHFc-F1 and sHFc-F2 diets resulted in a depression of body weight and the mass of epididymal fat. The DHA-rich sHFc-F1 diet exhibited a stronger effect compared to the EPA-rich sHFc-F2 diet (Table 6). Weight of dorsolumbar fat depot also tended to decrease, but the differences were not statistically

TABLE 4
Effects of EPA/DHA and CR on Fat Depots, Liver, and Food Consumption of Mice Fed cHF Diets

	Diet[a]				
	cHF	cHF-F1	cHF-F2	CR	cHF-F1 and CR
Epididymal fat					
Weight (mg of tissue)	1701 ± 124	1180 ± 190[b]	437 ± 60[b,c]	293 ± 60[b,c]	296 ± 50[b,c]
DNA (μg/mg of tissue)	0.39 ± 0.03	0.34 ± 0.04	0.74 ± 0.07[b,c]	0.76 ± 0.10[b,c]	0.65 ± 0.09[b,c]
(μg/depot)	652 ± 58	345 ± 47[b]	329 ± 63[b]	271 ± 50[b]	220 ± 20[b,c,d]
Dorsolumbar fat					
Weight (mg of tissue)	620 ± 40	552 ± 79	209 ± 16[b,c]	229 ± 24[b,c]	252 ± 14[b,c]
DNA (μg/mg of tissue)	0.56 ± 0.04	0.56 ± 0.05	1.20 ± 0.14[b,c]	1.41 ± 0.16[b,c]	1.05 ± 0.10[b,c]
(μg/depot)	323 ± 26	323 ± 40	244 ± 28[b]	348 ± 56	255 ± 15
Liver weight (mg)	1687 ± 85	1639 ± 74	1550 ± 120	915 ± 38[b,c,d]	935 ± 98[b,c,d]
Food consumption (kJ/d per animal)[e]	65 ± 3	NE	60 ± 3	NA	NA

[a]Animals described in Figure 1 were analyzed.
[b,c,d]Statistically significant differences compared to cHF, cHF-F1, and cHF-F2, respectively.
[e]Food consumption was estimated during the second week of feeding (week 2 in Fig. 1). There are different effects of diet on tissue weight and DNA content in various depots (ANOVA). CR, caloric restriction by 30% compared with *ad libitum*-fed mice; NA, not applicable; NE, not estimated.

TABLE 5
Effects of EPA/DHA on Body Weight and Fat Depots of Mice Fed sHFf Diet

	Diet[a]			
	Chow	sHFf	sHFf-F1	sHFf-F2
Body weight (g)				
Initial	25.5 ± 1.2	24.4 ± 0.9	25 ± 0.6	25.1 ± 0.8
Final	26.4 ± 0.8	24.3 ± 0.8	24.8 ± 0.6	22.2 ± 0.4[b,c]
Change	1.0 ± 0.4	0.6 ± 0.6	−0.15 ± 0.4	−2.9 ± 0.6[b,c]
Epididymal fat				
Weight (mg of tissue)	312 ± 45	357 ± 57	425 ± 52	182 ± 13[b,c]
DNA (μg/mg of tissue)	0.77 ± 0.07	0.77 ± 0.08	0.58 ± 0.04[b]	1.19 ± 0.14[b,c]
(μg/depot)	228 ± 24	213 ± 7	233 ± 11	211 ± 17
Dorsolumbar fat				
Weight (mg of tissue)	223 ± 18	236 ± 28	280 ± 18	207 ± 12[c]
DNA (μg/mg of tissue)	1.67 ± 0.26	1.22 ± 0.09	1.00 ± 0.04	1.57 ± 0.14[b,c]
(μg/depot)	352 ± 35	293 ± 21	279 ± 13	320 ± 23

[a]At 3 mon of age, chow diet-fed mice were divided into 4 groups (n = 8) and fed a chow, control sHFf, sHFf-F1 or sHFf-F2 diet. Animals were sacrificed at 5 months of age.
[b,c]Statistically significant differences compared to sHFf and sHFf-F1 diet, respectively. There are different effects of diet on tissue weight in various depots (ANOVA). See also Figures 2 and 3 for additional data from this experiment.

TABLE 6
Effects of EPA and DHA on Body Weight, Fat Depots, Lipid Content in Feces and Liver, and Plasma TG in Mice Fed sHFc Diets

	Diet[a]		
	sHFc	sHFc-F1	sHFc-F2
Body weight (g)			
Initial	26.9 ± 0.4	27.2 ± 0.5	27.1 ± 0.6
Final	28.1 ± 1.1	25.4 ± 0.7	26.2 ± 0.7
Change	1.7 ± 0.7	−1.4 ± 0.4[b]	−0.7 ± 0.6[b]
Epididymal fat weight (mg)	722 ± 132	355 ± 39[b]	450 ± 79
DNA (μg/mg of tissue)	0.51 ± 0.07	0.66 ± 0.08	0.74 ± 0.09
(μg/depot)	325 ± 26	216 ± 7.1[b]	339 ± 36
Dorsolumbar fat weight (mg)	268 ± 44	189 ± 12	205 ± 22
DNA (μg/mg of tissue)	0.76 ± 0.09	1.12 ± 0.11	1.29 ± 0.17[b]
(μg/depot)	190 ± 24	209 ± 19	253 ± 27
Food consumption (kJ/d per animal)[c]	73 ± 1	75 ± 2	73 ± 1
TG in feces (mg TG/g)	58 ± 9	76 ± 6	60 ± 7
TG in liver (mg TG/g)	159 ± 25	112 ± 6	91 ± 6[b]
Plasma TG (mg/dL)[d]	123 ± 9	49 ± 7[b]	36 ± 13[b]

[a]At 4 mon of age, chow diet-fed mice were divided into groups (n = 7) and fed by control sHFc, sHFc-F1, or sHFc-F2 diets. Animals were sacrificed at 6 and 1/2 mon of age.
[b]Statistically significant differences compared to sHFc diet.
[c]Mean food consumption during last 2 months of the experiment.
[d]At the time of sacrifice. In animals fed the control sHFf diet (see Table 5), food consumption was 78 ± 1 kJ/d per animal. There are different effects of diet on tissue DNA content in various depots (ANOVA).

significant (Table 6). Marked reduction of the epididymal fat due to sHFc-F1 diet was associated with a significant loss of tissue DNA (Table 6), indicating a reduction of tissue cellularity. On the other hand, the weak effect of sHFc-F2 diet on adiposity was accompanied by an increase in DNA concentration in both fat depots, but total DNA content in fat depots did not change. Food consumption was similar in all dietary groups (Table 6) including those fed the chow diet (not shown). Concentration of TG in feces was similar in all animals fed the sHF diets, whereas it was reduced in the liver of the sHFc-F2 compared with the sHFc mice (Table 6). Liver weight was similar in all groups of mice [data not shown; see also Hun *et al.* (7)].

Systemic consequences of dietary EPA/DHA. In the experiment, where the effects of EPA/DHA were tested using the sHFf diets, based on flaxseed oil (see Table 5), plasma levels of TG, NEFA, leptin, and insulin were also measured (Fig. 2). In parallel, the expression of leptin and GLUT4 genes in fat depots was also characterized (Fig. 3). Plasma TG levels were similar in mice fed the standard chow or sHFf diets, whereas they were decreased in proportion to the content of EPA/DHA in the diet. The levels of NEFA were similar in all groups of mice (Fig. 2). Leptin levels in plasma were greatly suppressed by the higher dose of EPA/DHA in the diet, whereas in all other dietary groups plasma leptin levels were similar (Fig. 2). The suppression of leptin concentrations by EPA/DHA

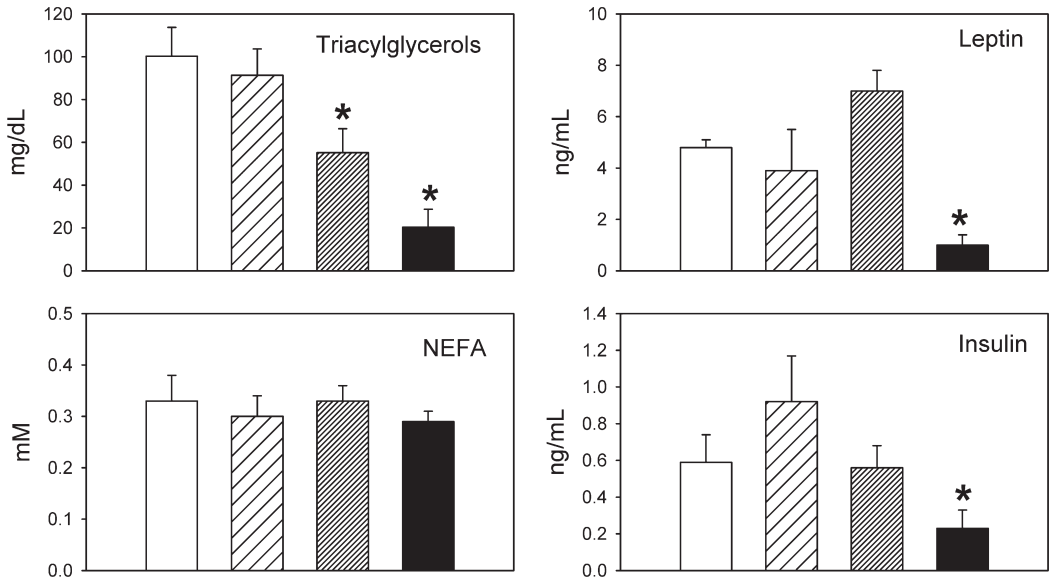

FIG 2. The effect of diet composition on plasma levels of TG, NEFA, leptin, and insulin. Metabolites and hormones were estimated in plasma of 5-mon-old mice fed either chow diet (open bars), control sHFf diet (coarse hatched bars), sHFf-F1 diet (fine hatched bars), or sHFf-F2 diet (filled bars). See Table 5 for further details. Data are means ± SE (n = 8). Asterisks indicate statistically significant differences compared with control sHFf diet.

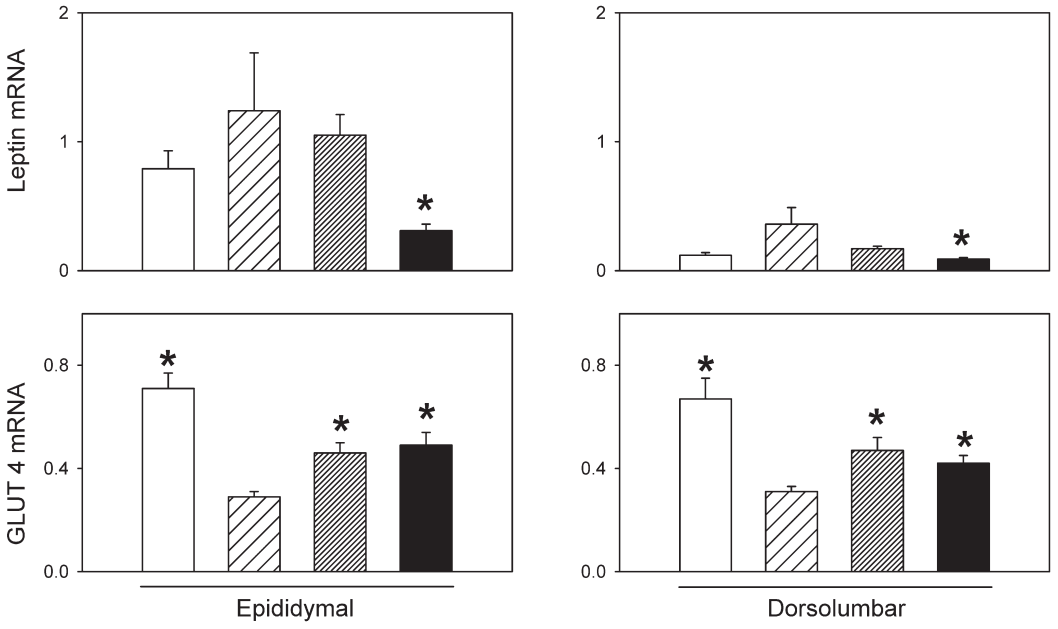

FIG 3. The effect of diet composition on transcript levels of leptin and GLUT4 in epididymal and dorsolumbar fat. Gene expression (arbitrary units) was estimated in total RNA isolated from different fat depots of 5-mon-old mice by using real-time quantitative PCR and standardized by using cyclophilin-β. For descriptions of the bars and other details, see Figure 2 and Table 5.

corresponded well with the changes in leptin gene expression in adipose tissue, namely, in epididymal fat, where the expression was in general much higher than in dorsolumbar fat (Fig. 3). The levels of insulin were similar in mice fed the control chow or sHFf diets, but were lowered proportionally to the content of the n-3 PUFA product in the diet (Fig. 2). On the contrary, blood glucose levels were not affected by dietary EPA/DHA (not shown). Expression of the GLUT4 gene, the insulin-regulated form of glucose transporters in adipocytes, was similar in both fat depots; however, it was about 2-fold higher in mice fed a standard chow compared to mice fed the sHFf diet. The expression was partially rescued by both sHFf-F1 and sHFf-F2 diets (Fig. 3).

In contrast to the differential effect of the sHFc diet rich in

DHA (sHFc-F1 diet) or EPA (sHFc-F2 diet) on weight and DNA content of the epididymal fat, plasma TG levels were lowered by both diets to a similar extent (Table 6).

DISCUSSION

In accordance with the previous studies (see introductory section) our results showed limited accumulation of epididymal fat by n-3 PUFA of marine origin admixed to various types of HF diets. Subcutaneous fat was less affected. During the development of obesity induced by cHF diet, accumulation of epididymal fat was significantly reduced when only 15% of dietary fat was replaced by lipids of marine origin and the content of EPA/DHA increased from about 1 to 12% (wt/wt) of dietary lipids. On the other hand, a larger part of dietary lipids had to be replaced by EPA/DHA for a significant reduction of adiposity in mice fed sHF diets, which did not promote obesity. A similar concentration of EPA/DHA in the sHF diet reduced adiposity also in rats (2,11). Extrapolation of our findings in mice fed cHF diets to an obese human, with a typical daily intake of about 100 g of dietary fat, suggests that additional daily intake of about 11 g of EPA/DHA would be required to limit obesity. In a clinical study conducted over 4 mon (13), daily fish meal (3.65 g of n-3 PUFA) in combination with a fat-restricted diet improved glucose metabolism and plasma lipid profile more effectively than the weight loss alone, whereas the combined treatment also tended to depress body weight more than the restriction of the dietary fat alone. In another clinical study, substitution of 6g/d of dietary fat by fish oil (1.8 g of n-3 EPA/DHA) resulted in a significant decrease of body fat over 3 weeks of treatment (15).

In accordance with the previous experiments (11), our study demonstrated that reduced accumulation of body fat in the EPA/DHA-treated animals did not result from a lower food consumption. The effect may be secondary to the stimulation of mitochondrial and peroxisomal FA oxidation in liver and muscle (2,9,11,13,28) and inhibition of hepatic lipogenesis and VLDL formation (1,2,4,9,13). These metabolic changes underlie the hypolipidemic effect of n-3 EPA/DHA, thus limiting the supply of FA to adipocytes. This idea is in accordance with our observation that an already low concentration of EPA/DHA contained in the sHFf-F1 diet, that did not affect adiposity, was sufficient to induce depression of plasma TG.

Development of obesity and accumulation of body fat may be also reduced by the modulation of gene expression in adipocytes. For example, EPA/DHA downregulate lipogenic genes (1,2,4,9,13) and stimulate expression of mitochondrial uncoupling proteins 2 and 3 (7,29) in adipose tissue. In our experiments, both DHA- and EPA-rich sHF diets (sHFc-F1 and sHFc-F2 diets, respectively) lowered plasma TG levels to a similar extent, whereas adiposity was preferentially decreased by the former diet (Table 4). These observations support the idea that the antiadipogenic effect does not result from the liver- and muscle-mediated hypolipidemia but depends, at least in part, on another mechanism. Changes in adipose tissue metabolism induced specifically by DHA could be involved. It has been observed before that natural fish oil (containing more EPA than DHA) was less potent in reducing the content of abdominal fat than an EPA/DHA mixture containing more of the latter PUFA (2).

The use of sHF diets in our experiments allowed us to characterize the significance of EFA, that is, ALA (18:3 n-3) and linoleic (18:2 n-6) acid for the antiadipogenic effect of EPA/DHA. ALA, abundant in the sHFf diets, is a precursor of EPA and DHA, but the efficiency of the conversion is low (see introductory section). Hence, dietary fish oil supplement is a more effective source of tissue DHA than is dietary ALA (4,14,30). In agreement with the above data, accumulation of epididymal fat was reduced by the sHFf-F2 diet, documenting that increased dietary intake of EPA/DHA reduces adiposity even in the presence of a high content of ALA. FA of the n-3 series are further metabolized to the group 3 series of eicosanoids, which could be involved in the antiadipogenic effect of EPA/DHA in adipocytes [for references, see Lapillonne *et al.* (9) and Raclot and Oudart (10)]. Linoleic acid (abundant in sHFc diets) serves as a precursor of arachidonic acid (20:4n-6) and groups 1 and 2 series of eicosanoids that promote adipogenesis [for references, see Azain (4), Massiera *et al.* (31), and Sessler and Ntambi (32)]. Competition between EFA occurs at the level of their desaturation and elongation since this metabolic pathway is shared by both FA. Therefore, abundance of ALA in the sHFf diets limits formation of arachidonic acid from linoleic acid and, in turn, inhibits the synthesis of the adipogenic eicosanoids of the n-6 series (30,31). Moreover, DHA inhibits cyclooxygenase, the key enzyme involved in the synthesis of these compounds (33). All these mechanisms could contribute to the reduction of fat storage due to EPA/DHA admixed to diets rich in ALA.

Enrichment of cHF diet with EPA/DHA at the low dose resulted in a depression of total amount of DNA in epididymal fat. Since each cell contains a constant amount of DNA, tissue DNA concentration and its amount could be used as markers of mean cell size and tissue cellularity, respectively. Apparently, the decrease of adipose tissue weight under these conditions was due to the reduction in number of cells in the tissue and not in the mean cell size. Only the higher dose of EPA/DHA increased DNA concentration in adipose tissue, indicating a decrease of mean cell size. DNA content of the epididymal fat was not affected by EPA/DHA-enriched sHFf diets. The different effect of EPA/DHA on DNA content and cellularity in animals fed the cHF (and also sHFc), compared with sHFf diets, could involve the inhibition of the conversion of arachidonic acid into proadipogenic eicosanoids by DHA, which probably occurs in sHFc and cHF diets. Such an effect of DHA may not take place in the sHFf diet since formation of arachidonic acid from linoleic acid in this diet is already inhibited by substrate competition with ALA (see previous discussion).

That the effect on cellularity was not noticed in the previous study (11) may be related to the fact that the number of mature fat cells rather than the content of DNA was evalu-

ated. However, DNA content of adipose tissue also decreases under other circumstances leading to depression of adiposity, such as the induction of energy dissipation by ectopic uncoupling protein 1 in adipocytes (34,35) or due to CR (3,36). Recent studies have shown a selective reduction of fat cell numbers by CR in male but not female rats (36) as well as stronger effect of CR on fat mass and gene expression in visceral than subcutaneous fat in humans (37).

Plasma leptin concentrations as well as leptin gene expression in epididymal fat positively correlated with adiposity under different dietary regimens, suggesting that leptin secretion from adipose cells followed the changes in adiposity [see also Hun *et al.* (7)]. Interestingly, EPA/DHA significantly reduced circulating insulin levels in a dose-dependent manner, suggesting improved whole-body insulin sensitivity and/or direct effect on pancreatic β-cells [see also Ikemoto *et al.* (6)]. The expression of glucose transporter GLUT4 partially restored in adipose tissue of mice treated with EPA/DHA suggests that improved insulin sensitivity of adipose tissue could be responsible, at least partly, for the enhancement in whole-body insulin sensitivity.

In summary, our results document augmentation of the anti-adipogenic effect of EPA/DHA during development of obesity and suggest that EPA/DHA could reduce accumulation of body fat by limiting both hypertrophy and hyperplasia of fat cells. Low EPA-to-DHA ratio potentiates the antiadipogenic effect. Increased dietary intake of EPA/DHA may be beneficial for prevention and treatment of obesity and related diseases regardless of ALA intake.

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